

# Circadian oscillation of gibberellin signaling in *Arabidopsis*

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Circadian clocks are endogenous timekeeping mechanisms that allow organisms to anticipate rhythmic, daily environmental changes. Temporal coordination of transcription results in a set of gene expression patterns with peak levels occurring at precise times of the day. An intriguing question is how a single clock can generate different oscillatory rhythms, and it has been proposed that hormone signaling might act in plants as a relay mechanism to modulate the amplitude and the phase of output rhythms. Here we show that the circadian clock gates gibberellin (GA) signaling through transcriptional regulation of the GA receptors, resulting in higher stability of DELLA proteins during daytime and higher GA sensitivity at night. Oscillation of GA signaling appears to be particularly critical for rhythmic growth, given that constitutive expression of the GA receptor expands the daily growth period in seedlings, and complete loss of DELLA function causes continuous, arrhythmic hypocotyl growth. Moreover, transcriptomic analysis of a pentuple *della* KO mutant indicates that the GA pathway mediates the rhythmic expression of many clock-regulated genes related to biotic and abiotic stress responses and cell wall modification. Thus, gating of GA sensitivity by the circadian clock represents an additional layer of regulation that might provide extra robustness to the diurnal growth rhythm and constitute a regulatory module that coordinates the circadian clock with additional endogenous and environmental signals.

crosstalk | plasticity | light | photoperiod

The pervasive role of the circadian clock driving plant physiology is reflected by the extensive regulation it exerts on gene expression, as more than one third of *Arabidopsis* genes are under circadian control (1). Remarkably, the expression of almost every single gene of *Arabidopsis* cycles when plants are grown under more realistic situations, for example combinations of thermo- and photocycles (2). This suggests that the entrainment of the circadian clock by light and temperature signals might allow plants to adapt to the daily changes in the environment by timing every physiological pathway to the specific time of day when it is more advantageous. For instance, the concerted action of the circadian clock and phyB-mediated light signaling allows the expression of a set of hormone-related genes toward dawn (3), which may provide robustness to the rhythmic patterns of growth of the seedling under diurnal conditions (4). Accordingly, a correlation exists between the oscillation of auxin-related genes and changes in the hypocotyl growth rate of seedlings grown under free-running conditions (5, 6), although the physiological significance of this correlation remains to be explored. In addition to light-mediated growth, the circadian clock controls the time of day that other environmental response pathways can be activated, often by triggering the oscillation of key signaling genes involved in these pathways (7). This type of regulation is known as gating because the clock can be thought of as opening or closing a gate to control the flow of information through a signaling pathway. Through such gating the circadian clock regulates many physiological responses including the photoperiodic induction of flowering and stress responses (8).

The phytohormone gibberellin (GA) has a prominent role in the regulation of several developmental programs also affected by light and the circadian clock, including the establishment of photomorphogenesis (9–11) and cell expansion (12), and the question arises of whether GA activity might mediate circadian regulation of clock targets. Given that such a regulatory mechanism would impact the robustness and flexibility of circadian regulation of development, we decided to explore this possibility and its physiological relevance.

## Results and Discussion

**Expression of GA Receptors Is Controlled by the Circadian Clock.** To investigate whether the circadian clock regulates GA signaling in *Arabidopsis*, we examined the daily expression pattern of all known GA signaling elements in the DIURNAL database (<http://diurnal.cgrb.oregonstate.edu/>) (2, 13). Although a weak oscillation could be detected in some cases, mostly linked to temperature rhythms, only the *GID1* receptor genes displayed robust cycling under short days (Fig. S14). The cycling of *GID1a* and *GID1b* was validated by real-time quantitative RT-PCR in independent time-course experiments, whereas we were not able to detect oscillation for *GID1c* transcript (Fig. 1A). The anticipation of changes in transcript levels to the light-to-dark and dark-to-light transitions and the oscillation under continuous light in entrained seedlings (Fig. S1B) suggested circadian rather than diurnal regulation. This was confirmed by analyzing mRNA levels in mutants defective for clock function, *toc1-1* (14) and *lhy* (15). The waveform of the oscillation in *toc1-1* seedlings was different from the WT, and the peak was narrower and was phased earlier (Fig. 1F). The phase advance is typical of *toc1-1* mutants, as a result of the deviation between their endogenous period (21 h) and the length of the day (24 h) (16). Transcript levels of both genes were altered also in the arrhythmic mutant *lhy* (Fig. 1K).

The expression of GA receptor genes is known to respond to endogenous GA levels through a DELLA-mediated feedback mechanism, i.e., their expression increases when GA levels are low and decreases when hormone levels are high (17). Hence, oscillation of *GID1* transcript levels might be a direct consequence of the circadian clock activity or, alternatively, it might respond to a putative oscillation of GA levels. However, *GID1a* expression was not altered in seedlings of the quadruple *della* mutant (*rga-t2 gai-t6 rgl1-1 rgl2-1*) (18, 19) (Fig. S2), indicating

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The authors declare no conflict of interest.

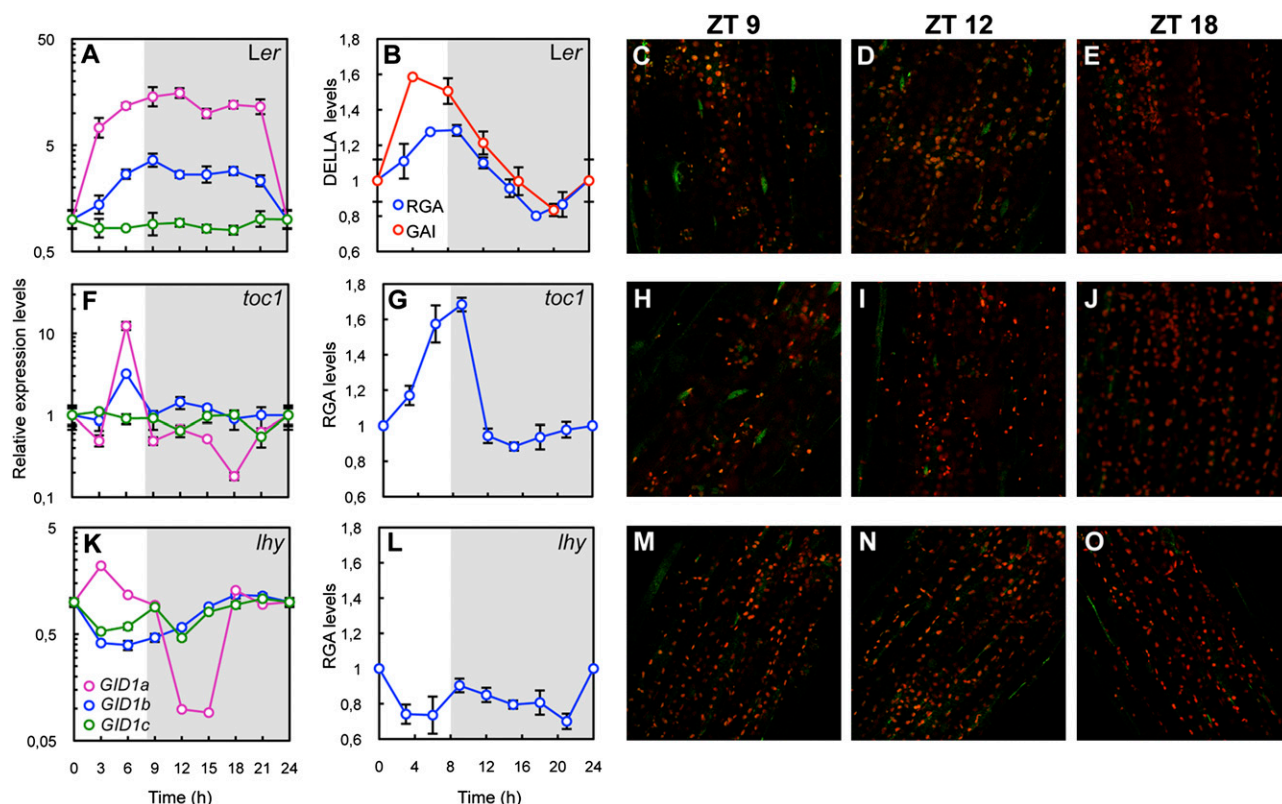
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Data deposition: The microarrays reported in this paper have been deposited in Nottingham *Arabidopsis* Stock Centre (code: NASCArray 587).

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that the circadian clock controls the expression of *GID1* genes independently of the status of the GA pathway.

**Levels of DELLA Proteins Oscillate with a Daily Rhythm.** GID1 receptors are known to interact with DELLA proteins in a GA-dependent way and promote their degradation (17). Thus, if the oscillation of GID1 expression is physiologically relevant, it should cause coherent changes in DELLA accumulation with a daily rhythm. We focused our attention in two DELLA proteins, GA INSENSITIVE (GAI) and REPRESSOR OF *gal-3* (RGA), which are the most abundant DELLAs in young seedlings and shoots (20). To monitor the level of these proteins, we used lines that express the *RGA::GFP-RGA* (21) or *35S::TAP-GAI* (22) transgenes. Consistent with the clock regulation of *GID1* genes, both GFP-RGA and TAP-GAI protein levels oscillated in a diurnal manner, showing peak levels at the end of the light period (Fig. 1B). Strikingly, GFP-RGA oscillation was detected in the growing region of the hypocotyls (Fig. 1C–E and H–J). Fluorescence from the fusion protein accumulated in nuclei of the uppermost part of hypocotyls at ZT9, whereas it was below the detection limit late in the night, at ZT18 (Fig. 1C–E), coinciding with periods of minimum and maximum growth rates, respectively (4). The periodicity of RGA accumulation must largely be caused by the activity of GID1 receptors, as the RGA transcript did not show significant oscillation (Fig. S1C). Thus, given (i) the major role of RGA and GAI in controlling growth (23, 24) and (ii) that seedling growth under diurnal conditions is

gated by the circadian clock (4), this result suggests that DELLA proteins are regulatory components for the control of the clock output, such as daily growth rhythm in young seedlings (4).

The oscillation of GFP-RGA levels was affected also in clock mutants. The waveform of GFP-RGA oscillation in *toc1-1* seedlings was slightly different from the WT, the amplitude was higher and the peak narrower because of an advance in the phase of the trough (Fig. 1 *G-J*), according to the phase advance observed in the expression of GA receptor genes (Fig. 1*F*). On the contrary, GFP-RGA protein levels were constant and low in the *lhy* mutant (Fig. 1 *L-O*), which correlates with the long hypocotyl phenotype observed in this mutant when grown in short days (3).

**Circadian Clock Gates GA Signaling Activity.** The observation that lower DELLA protein levels coincide with higher growth rates at the end of the night suggests that they participate in the core mechanism that controls rhythmic growth of hypocotyls. To test this hypothesis, we examined the impact upon growth of an alteration of the normal rhythm of GA signaling with two complementary approaches. First, we used a transgenic line that expresses a dominant version of GAI under the control of a heat-shock inducible promoter, *HS::gai-1D* (9). This line allowed us to block GA signaling by applying a 10-min heat shock at 33 °C at two different times of the day: ZT5, when the growth rate is low and DELLA levels high; and ZT17, which coincides with the beginning of the growing phase and with the trough of DELLA levels (Fig. 24). The effectiveness of the treatments was con-







tribute a more general role to DELLA proteins in the modulation of the output of the clock. Moreover, meta-analysis of the DELLA targets at ZT9 (Fig. 5C) indicated that only 45% of these genes were either direct targets for HY5 (30) or genes also regulated by the PIF transcription factors (31, 32). This implies that DELLA proteins control gene expression through the interaction with additional transcription factors, which is in agreement with the observation that DELLA proteins can interact with several members of the bHLH family of transcription factors other than PIFs (33, 34).

**Concluding Remarks.** The enormous plasticity of plant growth and development is based on a web of interacting signaling pathways, which provides the plant with multiple entry points to adjust their physiology in response to frequent, unpredicted environmental changes (35). The circadian system, on the contrary, provides the plant with the ability to anticipate predictable daily and seasonal environmental changes (36) and buffers plant responses against casual environmental variability (37). The circadian clock therefore provides stability to plants' life. Then, can responses regulated by the circadian clock be plastic? We suggest that the regulation of GA activity by the circadian clock might provide such ability, acting as a link between two properties critical for plant growth and development, robustness, and plasticity (38). Thus, the concurrency of clock and GA regulation of certain processes guarantees a precise and robust response to unpredicted, transitory, and above-noise changes in the environment that have an impact on the GA pathway, such as nutrient availability (39), salt stress (18), ambient temperature (40), or flooding (41). The observation that the functioning of the circadian clock does not seem to be affected significantly by GA (ref. 42 and the present study) supports the suggested role of GA as a regulatory output module that fine-tunes clock-regulated gene expression in response to environmental signals.

## Materials and Methods

**Plant Material.** *Arabidopsis thaliana* accessions Col-0 and Ler were used as WT. Seeds of *gid1a-1*, *gid1b-1*, *gid1c-1*, *gid1a-1 gid1b-1*, *gid1b-1 gid1c-1*, *gid1a-1 gid1c-1*, *toc1-1*, *lhy*, *rga-t2 gai-t6 rgl1-1 rgl2-1*, *rga-t2 gai-t6 rgl1-1 rgl2-1 rgl3-1*, *RG::GFP-RGA*, *35S::TAP-GAI*, and *HS::gai-1D* have been previously described (9, 15, 17, 18, 21, 22, 43). *RG::GFP-RGA toc1-1* and *RG::GFP-RGA lhy* lines were obtained by genetic crosses and isolated from an F<sub>3</sub> population.

**Plasmid Constructs and Transgenic Plants.** The preparation of transgenic lines expressing *35S::GID1a-YFP-HA*, *35S::GID1b-YFP-HA*, or *35S::GID1c-YFP-HA* was as follows. Coding sequences of *GID1a-1c*, excluding the stop codon, were PCR-amplified with Pwo polymerase (Roche) from cDNA obtained from 7-d-old, light-grown WT Col-0 seedlings. Oligonucleotides used as primers for PCR (Dataset S2) included the *attB* sites needed for Gateway-mediated cloning and were designed to allow expression of a C-terminal fusion. PCR products were first cloned into vector *pDONR-221* (Invitrogen) by BP recombination, and then transferred to the binary vector *pEarleyGate-101* (44) by LR recombination to create a C-terminal fusion with YFP and HA-tag. The final constructs were transferred to WT Col-0 plants by *Agrobacterium*-mediated transformation. Primary transformants were selected in MS plates containing 50 mM glufosinate ammonium (Fluka). Transgenic lines with a 3:1 (resistant:sensitive) segregation ratio were selected, and several homozygous lines were identified in the T<sub>3</sub> generation for each construction.

**Seedling Growth Assays.** All seeds were surface-sterilized with 70% (vol/vol) ethanol and 0.01% (vol/vol) Triton X-100 for 5 min, followed by 96% (vol/vol) ethanol for 5 min. Seeds were sown on plates of 0.5 MS medium (Duchefa) and 0.8% (wt/vol) agar without sucrose, and stratified at 4 °C in darkness for 5 d. Germination was induced by placing the plates under white fluorescent light (190–200  $\mu\text{mol m}^{-2} \text{s}^{-1}$ ) at 22 °C for 8 h. Seedlings were grown at 22 °C under short-day photoperiods, 8 h light (70–200  $\mu\text{mol m}^{-2} \text{s}^{-1}$ , depending on the experiment)/16 h dark in an E-30B growth cabinet (Percival).

For heat-shock experiments, WT Col-0 and *HS::gai-1D* (9) seedlings grown in the same plate under short days (70  $\mu\text{mol m}^{-2} \text{s}^{-1}$ ) received a heat treatment (10 min at 33 °C in darkness) at either ZT5 or ZT17. Control

seedlings of both genotypes were kept at 22 °C. Heat treatments were applied at days 3, 4, 5, and 6. Hypocotyl length was measured on day 7.

For GA sensitivity assays, *RG::GFP-RGA* seeds were sown on sterile filter papers placed on 0.5 MS, 0.8% (wt/vol) agar plates without sucrose, stratified, and induced to germinate as described earlier. After induction of germination, filter papers harboring seeds were transferred to treatment plates containing 0.2  $\mu\text{M}$  PAC (Duchefa) and grown under short days (190–200  $\mu\text{mol m}^{-2} \text{s}^{-1}$ ) at 22 °C for 3 d. Filter papers containing 4-d-old seedlings were transferred at either ZT0 or ZT12 for 1 h to Petri dishes with 5 mL of 0.5 MS liquid media containing 0.1  $\mu\text{M}$  GA<sub>4</sub> (Sigma) plus 0.2  $\mu\text{M}$  PAC, or just 0.2  $\mu\text{M}$  PAC. Filter papers containing seedlings were then rinsed three times for 20 min in Petri dishes containing 0.5 MS with PAC 0.2  $\mu\text{M}$ . After washes, seedlings were transferred into a new sterile filter paper, placed on a fresh 0.2- $\mu\text{M}$  PAC treatment plate, and returned to short-day conditions. GA treatments were given during two consecutive days (fourth and fifth), and hypocotyl length was measured on day 6. Handling of seedlings during the dark period was performed under a safe green light.

To measure hypocotyl length, seedlings were placed on an acetate sheet and scanned at a resolution of 600 dpi, and the length was measured with ImageJ software.

The time-lapse photography and image analysis to determine hypocotyl growth rate was performed as previously described (4), except that the growth medium contained 0.5 MS and 1% sucrose.

**RNA Extraction and Gene Expression Analysis by Quantitative RT-PCR.** Total RNA was isolated from whole seedlings grown as described earlier (190–200  $\mu\text{mol m}^{-2} \text{s}^{-1}$ ) by using the E.Z.N.A. Plant RNA Mini Kit (Omega Bio-tek) according to the manufacturer's instructions. cDNA synthesis and quantitative PCR conditions were as described (9). Primers used are listed in Table S2.

**Microarray Analysis.** WT Ler and *rga-t2 gai-t6 rgl1-1 rgl2-1 rgl3-1* pentuple *della* mutant seedlings were grown under short days (190–200  $\mu\text{mol m}^{-2} \text{s}^{-1}$ ) at 22 °C, and seedlings were sampled at ZT9 and ZT12 of day 5. Total RNA from whole seedlings was extracted with RNeasy Plant Mini kit (Qiagen). RNA labeling and hybridization to Affymetrix *ATH1* arrays were performed by the Nottingham *Arabidopsis* Stock Centre. Analysis was performed in R (45) and Bioconductor (46). Microarrays were normalized with the RMA procedure as implemented in the affy package (47), and differential expression was determined using limma (48, 49) with a false discovery rate lower than 0.05. To determine genes whose expression varies in diurnal short-day conditions, the previously published data set (13) was downloaded from Array Express (<http://www.ebi.ac.uk/arrayexpress/>; accession E-MEXP-1304), Robust Multichip Average-normalized, and analyzed in limma by using a one-way ANOVA model with time as the grouping variable. Samples from each of the 2 d of collection were used as replicates (so, in total, there were two replicates for each of six time points). Gene annotations were based on the TAIR9 version of the *Arabidopsis* Web site (<http://arabidopsis.org>). Gene Ontology analysis was performed with the Classification Super-Viewer tool at the Bio-Array Resource of the University of Toronto.

**Protein Extraction and Western Blot Analysis.** Protein extraction and Western blot analysis from whole 5-d-old seedlings grown under short days (190–200  $\mu\text{mol m}^{-2} \text{s}^{-1}$ ) were performed as described. The GFP, TAP, and HA fusion proteins were detected using anti-GFP (JL8; Clontech), anti-c-myc (9E10; Roche), and anti-HA (3F10; Roche) antibodies, respectively. Antibodies against DET3 were used to check protein loading (50). Signal from bound antibodies was revealed using ECL Advance Western Blotting detection Kit (GE Healthcare) and visualized and quantified by using the Luminescence Image Analyzer LAS-3000 (Fujifilm) and Image Gauge (version 4.0; Fujifilm), respectively.

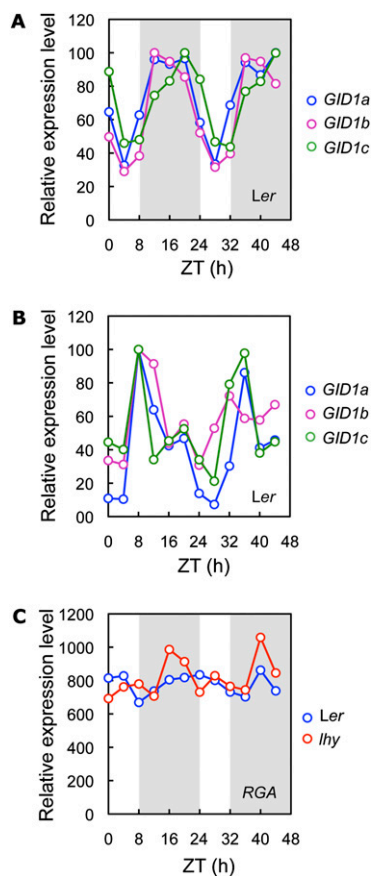
**Confocal Imaging.** Fluorescence from the GFP-RGA fusion protein was detected by using a confocal microscope (TCS SL; Leica) as previously described (40).

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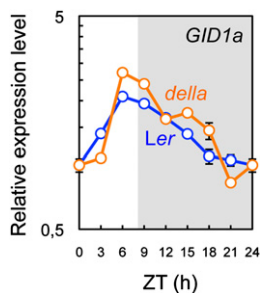
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# Supporting Information

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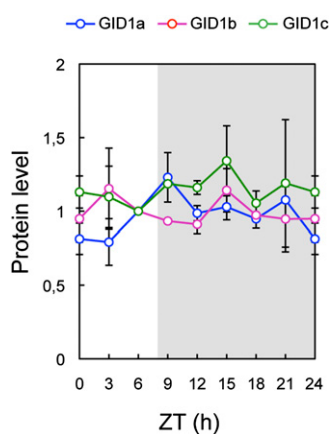
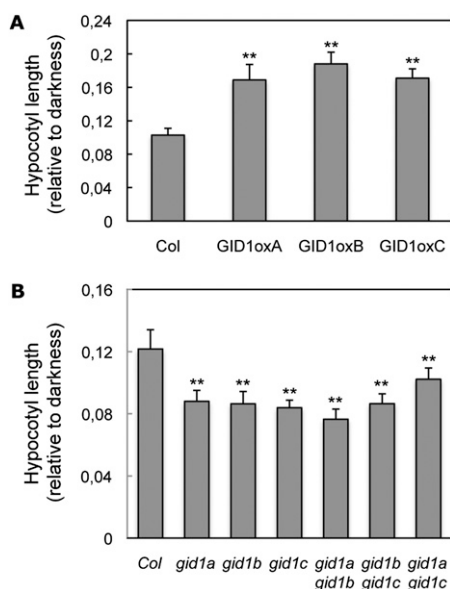


**Fig. S1.** The circadian clock controls diurnal oscillation of *GID1* genes in *Arabidopsis*. (A) Transcript levels of *GID1* genes in short-day photoperiods (8 h light/16 h dark). (B) Transcript levels of *GID1* genes in continuous light after entrainment in short-day photoperiods. (C) Expression of *RGA* in WT and *lhy* mutant seedlings in short-day photoperiods. Data are taken from DIURNAL (<http://diurnal.cgrb.oregonstate.edu/>) and are normalized to the average value to facilitate comparison.



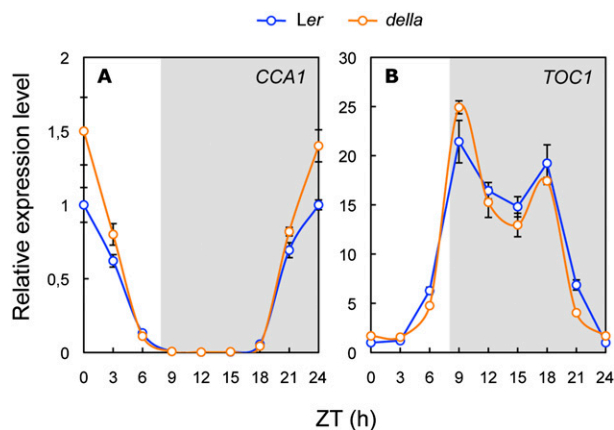
**Fig. S2.** Expression of *GID1a* in 5-d-old *Ler* WT and in quadruple *della* mutant seedlings grown under short-day photoperiods (8 h light/16 h dark). Values are expressed relative to *PP2a* expression. Data represent mean  $\pm$  SD of three technical replicates. Experiments were repeated twice with similar results. White and gray areas represent day and night, respectively.





**Fig. S4.** GID1s accumulate constitutively in the overexpressing lines. *35S::GID1a*, *35S::GID1b*, and *35S::GID1c* seedlings were grown for 5 d in short-day photocycles (8 h light/16 h dark). GID1-GFP protein levels were determined by Western blot with commercial antibodies against GFP. GID1 levels were normalized against levels of DET3, which was used as loading control. Data are the mean of two independent experiments. White and gray areas represent day and night, respectively.





**Fig. S5.** The core clock mechanism is not affected in the pentuple *della* mutant. WT Ler and pentuple *della* seedlings were grown under short-day photocycles (8 h light/16 h dark) for 5 d. Expression of *CCA1* (A) and *TOC1* (B) clock genes is shown. Values are expressed relative to *PP2a*. Data represent mean and SD of three technical replicates. Experiments were repeated twice with similar results; results from one representative experiment are shown. White and gray areas represent day and night, respectively.

## Other Supporting Information Files

Dataset S1 (XLS)

[Dataset S2 \(XLS\)](#)